



First report of the land planarian *Diversibipalium multilineatum* (Makino & Shirasawa, 1983) (Platyhelminthes, Tricladida, Continenticola) in Europe

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Introduction of alien species may significantly affect soil ecosystems, through predation or disruption of components of native ecosystems (Winsor *et al.* 2004; Álvarez-Presas *et al.* 2014; Justine *et al.* 2014). Land planarians have been reported as alien species in soils throughout the world and, among those, some species are considered to be successful invaders, e.g. *Platydemus manokwari* de Beauchamp, 1963, *Arthurdendyus triangulatus* (Dendy, 1894), *Bipalium adventitium* Hyman, 1943, *Bipalium kewense* Moseley, 1878 and *Dolichoplana striata* Moseley, 1877 (Winsor *et al.* 2004; Álvarez-Presas *et al.* 2014; Justine *et al.* 2014, 2015). Soil moisture status seems to be an important element for their successful invasion (Fraser & Boag 1998). In Europe at least 18 species of alien land planarians have been recorded since now and some of them are considered as invasive ones, e.g. *P. manokwari* (cf. Justine *et al.* 2014). Although the alien land planarian *B. kewense* has been reported to occur in many greenhouses in Italy (Bello *et al.* 1995), no data are available on its establishment and/or impact on natural environments. On 28th September 2014, 20 specimens (~1 individual/m²) of the land planarian *Diversibipalium multilineatum* (Makino & Shirasawa, 1983) (Fig. 1), native to Japan, were collected under pots, branches and plastic materials in a private garden located in the center of Bologna (Emilia Romagna, Central Italy), near the urban park Giardini Margherita (44°29' N, 11°21' E; WGS84). Thirty plant species (both indigenous and alien), mainly cultivated as bonsai (e.g. *Lagerstroemia indica* L., *Juniperus procumbens* (Siebold ex Endl.) Miquel), were present in this shady, wet garden (25 m²). Between March 2014 and June 2015, 70 more specimens of *D. multilineatum* were collected at the same site, mainly at dusk and dawn after rain. Reproduction by fission and regeneration processes were observed in several of those specimens, which were kept for some time in captivity. A specimen of *D. multilineatum* was also collected in a garden in Lègevin (Haute-Garonne, France), which will be described in a forthcoming paper by Justine *et al.* (in prep.) (see also Kawakatsu *et al.* 2014). Specimens without a genital pore were initially ascribed to *D. multilineatum* on the basis of their external appearance: the dorsal surface was brownish yellow and presented five longitudinal stripes at the head plate and the neck, showing the typical appearance of the species. The middorsal stripe was widened at its anterior end, on the head plate, and at the pharynx level. The ventral pattern of the animals at the pharyngeal region was also characteristic, with the middorsal stripe widened at this level. The Italian *Diversibipalium* specimens used for the molecular analysis were fixed and preserved in absolute ethanol. Fragments of the mitochondrial gene COI and 28S ribosomal RNA nuclear gene (GenBank Acc. Numbers KU245358 and KU245357, respectively) were obtained using the procedure and COI primers described in Álvarez-Presas *et al.* (2008) and Solà *et al.* (2013). The French specimen's COI (Specimen MNHN JL177, GenBank Acc. Number KT922162) was obtained as described in Justine *et al.* (2015). 28S sequences of 14 Bipaliinae specimens and four

Microplana species (outgroup) retrieved from GenBank were included in the phylogenetic analyses (Fig. 2). Sequence alignment was obtained by using the online software MAFFT version 7 (Katoh & Standley 2013), while ambiguously aligned positions were removed using the program Gblocks (Talavera & Castresana 2007) with default settings, excepting the minimum number of sequences for a flank position at the minimum value (set at 10) and with half of the allowed gap positions. The final alignment had a length of 1589 bp. We used two phylogenetic inference approaches: maximum likelihood (ML), using the RaxML 8.2.3 software (Stamatakis 2014), and Bayesian inferences (BI), using MrBayes 3.2.4 (Ronquist *et al.* 2012). The evolutionary model used, GTR+I+G, was estimated to be the best with the software jModeltest 2.1.7 (Darriba *et al.* 2012; Guindon & Gascuel 2003), using the Akaike Information Criterion (AIC). MrBayes analyses were performed for 10-million generation with sampling parameters every 10^3 and a 25% default burn-in value for the final trees. Convergence of the two runs (average standard deviation of split frequencies $\ll 0.01$) and likelihood stationarity were checked. The maximum likelihood analyses were performed under 1000 bootstrap pseudoreplicates. The phylogenetic results show a close and highly supported relationship of the Italian *Diversibipalium* specimens with those from Japan and South Korea that have been identified as *D. multilineatum* (Fig. 2). *Diversibipalium multilineatum* is the sister-group of *B. nobile* Kawakatsu & Makino, 1982, but with low support. The COI sequences of the French (MNHN JL177) and the Italian *Diversibipalium* specimens were compared in Geneious v. 8.0.5 (<http://www.geneious.com>, Kearse *et al.* 2012) and were found to be identical. These results indicate that the species introduced in both countries is the same, and most probably concerns the species *D. multilineatum*. The pathways of introduction of *D. multilineatum* are currently unknown, although a relationship between the horticultural trade and the introduction of alien land planarians is well known (Álvarez-Presas *et al.* 2014 and references therein). Here we report the first occurrence of individuals of *D. multilineatum* outside Asia. The GenBank sequence of *D. multilineatum* from South Korea is not yet supported by a published description of the specimen, while it is debatable whether South Korea should be considered part of the natural range of *D. multilineatum*, which only seems to include Japan. In the present paper, we consider the South Korean animal to be an introduced specimen. Soil moisture status, temperature, and food availability are considered to be the main factors determining the presence of terrestrial planarians (Boag *et al.* 1998); the microclimatic conditions of the Italian garden were similar to plant nurseries and greenhouses, while an abundance of food was available, such as isopods [*Porcellionides pruinosus* (Brandt, 1833)], oligochaetes [*Dendrobaena attemsi* (Michaelsen, 1902) and several juveniles of *Lumbricus* spp.] and gastropods [*Cermtella cisalpina* (Rossmassler, 1837), *Cornu aspersum* (O.F. Müller 1774), *Deroceras reticulatum* (O.F. Müller, 1774), *Discus rotundatus* (O.F. Müller, 1774), *Limacus flavus* (Linnaeus, 1758), *Milax nigricans* (Philippi, 1836), *Papillifera papillaris* (Linnaeus, 1758), *Pomatias elegans* (O.F. Müller, 1774)]. Moreover, winter 2014 reached the highest temperatures and rainfall of the last two decades (source: CNR-ISAC, Bologna), thus favouring establishment and spread of *D. multilineatum*. The potential environmental impacts of some invasive flatworms are well documented (Álvarez-Presas *et al.* 2014; Justine *et al.* 2014) and, even if these effects have not yet been assessed for *D. multilineatum*, the adoption of precautionary measures and of early intervention is here strongly recommended (Genovesi & Shine 2004). Finally, knowledge of the introduction pathway(s), together with the analysis of prey preference and possible impact on the invertebrate fauna, will be essential to halt or at least to limit the spread of this introduced land flatworm.

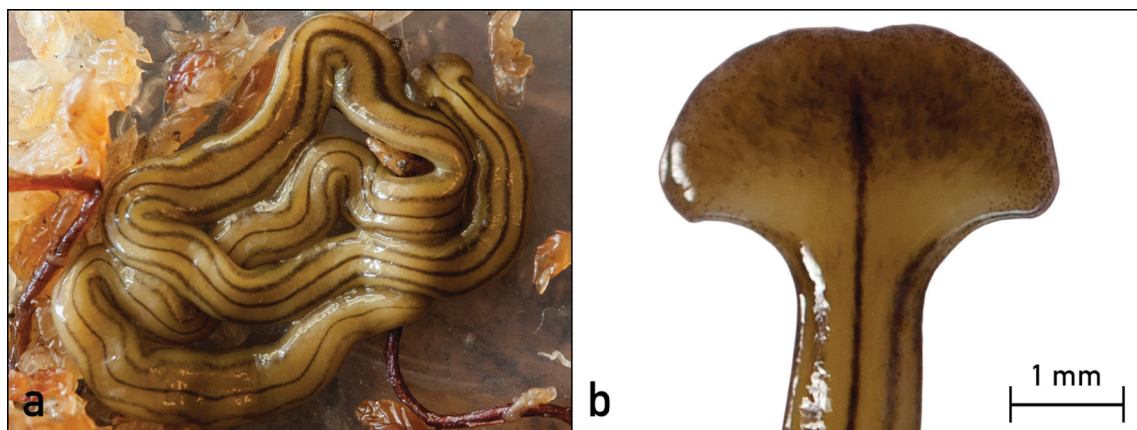


FIGURE 1. A specimen of *Diversibipalium multilineatum* (Makino & Shirasawa, 1983) collected from a private garden in Bologna (Italy).

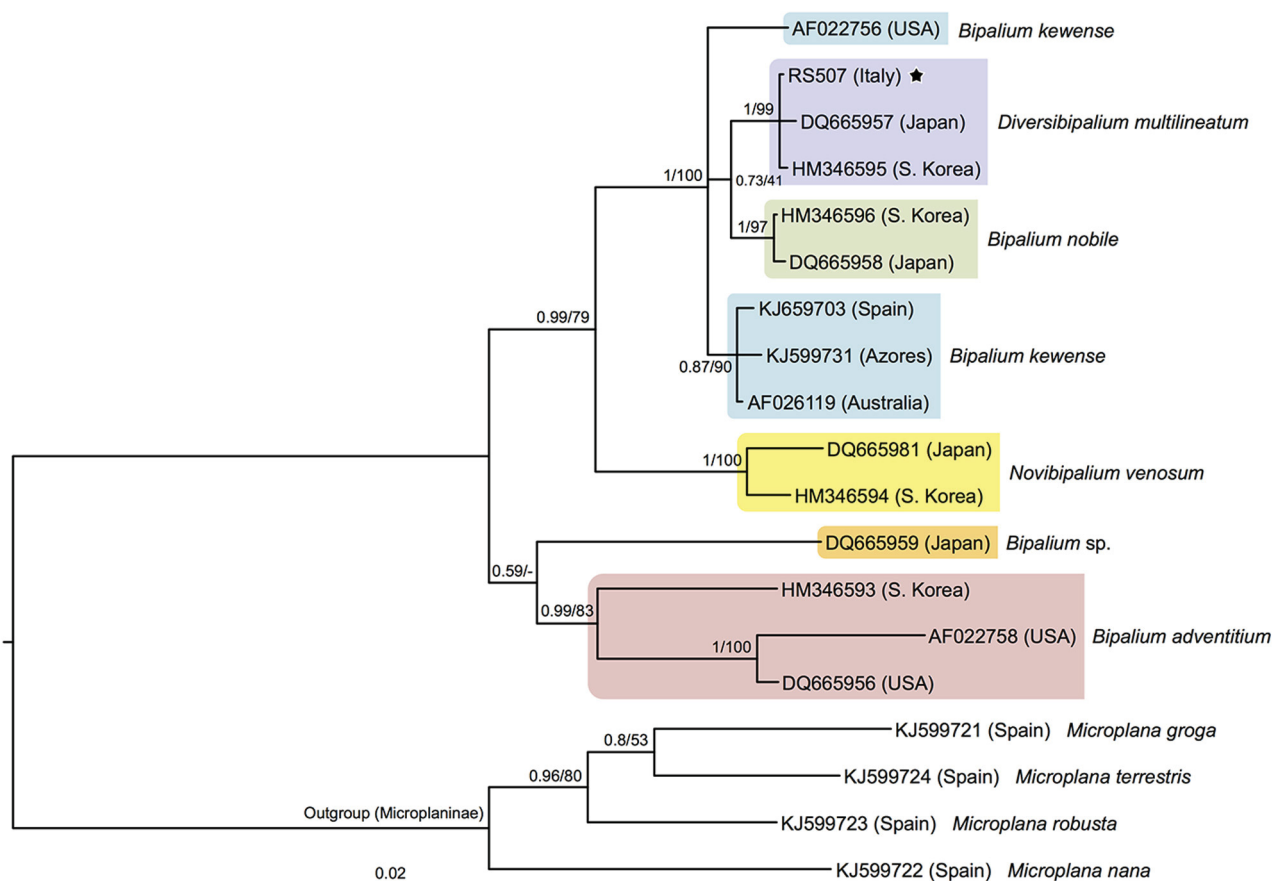


FIGURE 2. Bayesian tree inferred from 28S data set. Labels correspond to GenBank Accession Numbers. Node numbers correspond to posterior probability (BI)/bootstrap (ML). The scale bar indicates substitutions per site.

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